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(54) Title: METHOD FOR PRESERVING LIPOSOMES

(57) Abstract

A novel method is provided for preserving liposomes containing biologically active molecules, such that when rehydrated, liposome structures retain substantially all material originally encapsulated. A preserving agent having at least two monosaccharide units is used either internally or externally or both. In a preferred embodiment, trehalose is used as a preserving agent, both inside the liposomes as an encapsulate material and externally, in solution, during freeze-drying. The invention also includes a lyophilized composition prepared by the disclosed method.

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METHOD FOR PRESERVING LIPOSOMES

Field of the Invention

The present invention relates generally to liposomes, and more particularly relates to a method of preserving liposomes containing biologically active molecules. This process is useful in applications such as in vivo drug delivery and preservation of diagnostic agents.

This invention was made with Government support under Grant No. PCM 82-17538 with the National Science Foundation and the University of California. The Government has certain rights in this invention.

Background of the Invention

Liposomes are unilamellar or multilamellar lipid vesicles which enclose a fluid space. The walls of the vesicles are formed by a bimolecular layer of one or more lipid components having polar heads and non-polar tails. In an aqueous (or polar) solution, the polar heads of one layer orient outwardly to extend into the surrounding medium, and the non-polar tail portions of the lipids associate with each other, thus providing a polar surface and a non-polar core in the wall of the vesicle. Unilamellar liposomes have one such bimolecular layer, whereas multilamellar liposomes generally have a plurality of substantially concentric bimolecular layers.

Liposomes are well recognized as useful for encapsulation of drugs and other therapeutic agents and for carrying these agents to in vivo sites. For example,

U.S. Patent 3,993,754, inventors Rahman et al., issued November 23, 1976, discloses an improved chemotherapy method in which an anti-tumor drug is encapsulated within liposomes and then injected. U.S. Patent 4,263,428, inventors Apple et al., issued April 21, 1981, discloses an antitumor drug which may be more effectively delivered to selective cell sites in a mammalian organism by incorporating the drug within uniformly sized liposomes. Drug administration via liposomes can have reduced toxicity, altered tissue distribution, increased drug effectiveness, and an improved therapeutic index.

Liposomes have also been used successfully for introducing various chemicals, biochemicals, genetic material and the like into viable cells in vitro, and as carriers for diagnostic agents.

A variety of methods for preparing liposomes are known, many of which have been described by Szoka and Papahadjopoulos, Ann. Rev. Biophysics Bioeng. 9: 467-508 (1980). Also, several liposome encapsulation methods are disclosed in the patent literature, notably in U.S. patent 4,235,871, to Papahadjopoulos et al., issued November 25, 1980, and in U.S. patent 4,016,100 to Suzuki et al., issued April 5, 1977.

Although encapsulation of therapeutic agents and biologically active compounds in liposomes has significant commercial potential, a major difficulty that has been encountered in the commercial use of liposome encapsulates is with their long term stability. Although liposome structures may be maintained intact under certain storage conditions, such conditions are often inconvenient or unavailable. It is as a solution to this problem that the method of this invention is presented.

Summary of the Invention

Accordingly, it is an object of the present invention to provide a commercially feasible method of preserving liposomes.

It is another object of the present invention to provide a commercially feasible method of preserving liposomes by means of freeze-drying, wherein upon rehydration, resultant liposomes can retain as much as 100% of their original encapsulated material.

It is still another object of the present invention to provide a method of preserving liposomes by means of a carbohydrate compound capable of preserving structure and function in biological membranes.

It is a further object of the present invention to provide a method of preserving liposomes by means of a preserving agent such as trehalose, present either as encapsulated material inside the liposome or externally in solution during freeze-drying, or both.

It is yet another object of the present invention to provide a lyophilized composition which upon rehydration retains up to 100% of original encapsulated material.

Further objects and advantages of this invention will become apparent from the study of the following portion of the specification, the claims and the attached drawings.

In one aspect of the present invention, a method for preserving liposomes includes freeze-drying liposomes in the presence of a preserving agent capable of preserving structure and function in biological membranes. Preferred preserving agents include carbohydrates having at least two monosaccharide units, and especially preferred compounds include the disaccharides

sucrose, maltose, and trehalose.

In another aspect of the present invention, the method comprises freeze-drying liposomes which in addition to containing biologically active molecules or therapeutic agents contain a preserving agent such as trehalose internally. In a preferred embodiment of the inventive method, an appropriate compound such as trehalose is present both inside and outside the lipid membrane; preferred weight ratios of total preserving agent to lipid range from about 0.1:1 to 3.0:1. An especially preferred weight ratio is about 1.0:1.0.

The invention also embodies a lyophilized composition such that when reconstituted by rehydration, resultant liposomes retain substantially all of their originally encapsulated material. Such a lyophilized composition may be prepared by the method as outlined above.

Detailed Description of the Invention

The invention comprises a method for preserving liposomes containing biologically active molecules using a preserving agent. The method involves either freeze-drying liposomes in the presence of a preserving agent, or freeze-drying liposomes which contain a preserving agent internally in addition to encapsulated medicaments, or both. Preferred preserving agents are carbohydrates having at least two monosaccharide units joined in glycosidic linkage, and particularly preferred preserving agents include sucrose, maltose and trehalose. Of these, trehalose has been found to be the most effective preserving agent for use with the inventive method.

Trehalose is a naturally occurring sugar found

at high concentrations in organisms capable of surviving dehydration. Trehalose is especially effective in preserving structure and function in dry biological membranes. Liposomes which are freeze-dried in the presence of trehalose and which additionally contain encapsulated trehalose, exhibit particularly good retention of encapsulates. That is, when liposomes are exposed to trehalose both internally and externally during freeze-drying, they can retain as much as 100% of their original encapsulated contents upon rehydration. This is in sharp contrast to liposomes which are freeze-dried without any preserving agent, which show extensive fusion between liposomes and loss of contents to the surrounding medium.

Representative phospholipids used in forming liposomes which may be used in this process include phosphatidylcholine, phosphatidylserine, phosphatidic acid and mixtures thereof. Both natural and synthetic phospholipids may be successfully used.

The biologically active or therapeutic encapsulated material is preferably water soluble. Examples of suitable therapeutic agents with which this preservation method can successfully be carried out include sympathomimetic drugs such as amphetamine sulfate, epinephrine hydrochloride, or ephedrine hydrochloride; antispasmodics such as atropine or scopalamine; bronchodilators such as isoproternol; vasodilators such as dilthiazen; hormones such as insulin; and antineoplastic drugs such as adriamycin. Suitable biologically active molecules include, for example, RNA, DNA, enzymes and immunoglobulins.

Small unilamellar vesicles (SUV's) are prepared as starting materials prior to encapsulation of trehalose, and may be prepared by any of the available

techniques. Suitable techniques include injection of the lipid in an organic solvent into water, extrusion from a French pressure cell, and sonication. The material to be trapped may be added at any stage during preparation of the small unilamellar vesicles, but in practice it is most convenient to mix the small unilamellar vesicles with an aqueous solution of the material to be trapped immediately before preparation of large unilamellar vesicles. Preferred weight ratios of encapsulate to lipid are about 1.0:1.0.

Large unilamellar vesicles (LUV's) with increased trapping efficiency may then be prepared by either freeze-thawing or rotary evaporation. An exemplary rotary evaporation method and one which is especially effective in conjunction with the method disclosed herein is illustrated in Deamer, D.W., "A Novel Method for Encapsulation of Macromolecules in Liposomes" in Gregoriadis, G. (ed.) Liposome Technology (1984). The method comprises providing a polar solution having initial liposomes and a quantity of material to be encapsulated. Substantially all of the solution is removed, and the resultant liposomes are then recovered by hydration of the concentrated admixture. This method is also the subject of pending U.S. Patent Application Serial No. 493,952, inventor Deamer, et al., filed May 12, 1983. The resulting vesicles may then be made more uniform by filtration, centrifugation or gel permeation chromatography.

Trehalose may be added at any stage during preparation of the large unilamellar vesicles, but greatly improved preservation is attained with trehalose present on both sides of the phospholipid bilayer. Therefore, trehalose is preferably added before the large

unilamellar vesicles are prepared, so that trehalose is trapped inside. The preferred weight ratio of total trehalose to lipid ranges from about 0.1:1 to about 3.0:1; a particularly preferred weight ratio is approximately 1.0:1.0. The large unilamellar vesicles are then frozen in liquid nitrogen and lyophilized. Under some circumstances, as when lipids are used which are susceptible to damage due to the presence of oxygen, it may be desirable to seal the dry preparations under vacuum. Rehydration is accomplished simply by adding water to the dry mixture.

Although in a preferred embodiment of the invention, the liposomes are exposed to trehalose, it should be understood that a variety of preserving agents may be substituted for trehalose, including carbohydrate compounds which are composed of at least two monosaccharide units. In particular, sucrose and maltose are suitable alternatives.

The following examples illustrate certain aspects and embodiments of the present invention, and are not intended to limit the scope of the invention as defined in the appended claims.

Example 1

A phopholipid mixture consisting of approximately 40 mg. dipalmitoyl phosphatidylcholine and phosphatidic acid in a molar ratio of 95:5 was sonicated to optical clarity in a bath sonicator. Large unilamellar vesicles were prepared by freeze-thawing in a 50 mM solution of isocitric acid in water as the compound to be encapsulated. Excess isocitric acid was removed by dialysis. Trehalose (2.0:1.0 trehalose:phospholipid weight ratio) was added either after freeze-thawing or beforehand, thus providing some large unilamellar vesicles with external

trehalose only and some vesicles with trehalose both externally and internally. Isocitric acid was assayed by adding isocitrate dehydrogenase and NADP to the outside of the vesicles according to the method of Plaut, et al. (Eds.), Methods in Enzymology, Volume 5 (New York: Academic Press). Isocitrate external to the vesicles was oxidized by the isocitrate dehydrogenase, resulting in reduction of NADP to NADPH, the rate and amount of which may be recorded fluorometrically. Total isocitric acid in the vesicles was assayed following addition of Triton X-100 (octylphenoxy polyethoxyethanol, a detergent and emulsifier manufactured by Rohm & Haas Co., Philadelphia, PA; "TRITON" is a registered trademark of Rohm & Haas Co.), which releases the trapped isocitric acid into the surrounding medium. Isocitric acid trapped in the vesicles was assayed before and after both lyophilization and rehydration, thus providing an estimate of the efficiency with which the trapped isocitrate was retained. As may be seen in Table 1, the results show that over sixty percent (60%) of the trapped isocitrate was retained when the vesicles were lyophilized with trehalose both inside and outside the vesicles. When trehalose was present externally only, there was still a significant increase in the efficiency of retention, but to a lesser degree than in the case where trehalose was present on both sides of the lipid membrane. Examination of lipid concentration at time of freezing showed that such had no significant effect on retention of trapped material following lyophilization.

Table 1

Method of Preparing LUV's	Concentration of Lipid (mg/ml)	g Trehalose /g Lipid	Trehalose External	Trehalose Internal	%Retention
FT*	10.8	0	-	-	0
FT	11.1	0.08	-	+	0
FT	10.8	1.78	+	-	42
FT	11.1	1.78	+	+	61

*FT = freeze-thaw

Example 2

Small unilamellar vesicles of were made by sonication of 43 mg egg phosphatidylcholine in 4 ml of water. Large unilamellar vesicles were prepared by rotary drying the phospholipid in the presence of 32 mg of trehalose and 13 mg of isocitric acid. The weight ratios of phospholipid:trehalose:isocitric acid were approximately 4:3:1. Excess isocitric acid and trehalose were removed by dialysis against distilled water, and the amount of isocitric acid trapped in the vesicles was determined by the enzyme assay described in Example 1. Trehalose was added to the dialyzed liposomes to give a final weight ratio of phospholipid:trehalose of 1.0:1.4, and the sample was lyophilized. The sample was then rehydrated with distilled water, and the amount of isocitric acid remaining in the liposomes was determined by enzyme assay. The lyophilized vesicles retained 75% of their original contents.

Example 3

A phospholipid mixture of palmitoyloleoyl phosphatidylcholine (90%) and phosphatidylserine (10%) was hydrated to 10 mg./ml., and small unilamellar vesicles were then prepared by sonication. Large unilamellar vesicles were prepared by rotary drying in the presence of isocitric acid, which served as the encapsulated molecule. Essentially the same techniques as previously described in Examples 1 and 2 were used. Efficiency of retention of isocitric acid following lyophilization and rehydration was recorded as before, with large unilamellar vesicles lyophilized first in the presence and then in the absence of trehalose. As may be seen in Table 2, the results show that 100% of the trapped

isocitric acid is retained when the large unilamellar vesicles are lyophilized and rehydrated under the stated conditions. As the previous examples demonstrated, trehalose is preferably present both externally and internally to optimize retention of the encapsulate.

Example 4

One of the damaging events presumed to be occurring during lyophilization is close approach of the large unilamellar vesicles to each other, leading to fusion and leakage of the vesicular contents. Fusion has been assayed by resonance energy transfer, a fluorescence method which depends upon energy transfer from an excited probe (the "donor probe") to a second probe (the "acceptor probe"). The acceptor probe fluoresces when the energy transfer occurs. In order for the transfer to occur the two probes must be in close proximity. Thus probe intermixing can be used as an assay for fusion between vesicles during lyophilization. Large unilamellar vesicles were prepared with donor probe in one preparation and acceptor probe in another, and the two preparations were mixed before lyophilization. Following lyophilization and rehydration, probe intermixing was measured, with the results listed in Table 3. The results show that with increasing trehalose concentration there is a decrease in probe intermixing. Furthermore, the presence of trehalose inside the liposomes alone significantly reduce probe mixing. Thus, use of trehalose tends to reduce fusion of the vesicles.

Table 2

Method of Preparing LUV's	g Trehalose /g Lipid	Trehalose External	Trehalose Internal	%Retention
RD*	0.06	-	+	0
RD	3.2	+	+	100
RD	0	-	-	0
RD	3.9	+	-	26
RD	0.11	+	+	22
RD	0.19	+	+	49
RD	0.33	+	+	69
RD	0.63	+	+	76
RD	0.91	+	+	86
RD	1.76	+	+	99

*RD = rotary drying

Table 3

Method of Preparing LUV's	g Trehalose /g Lipid	Trehalose External	Trehalose Internal	% Probe Mixing
RD*	0.05	-	+	72
RD	0.15	+	+	39
RD	0.25	+	+	29
RD	0.50	+	+	12
RD	0.95	+	+	8
FT**	0.	-	-	93.0
FT	0.4	+	+	79.0
FT	0.8	+	+	59.0
FT	1.2	+	+	54.0
FT	1.6	+	+	38.0
FT	2.0	+	+	15.0

*RD = rotary drying

**FT = freeze-thaw

FUSION BETWEEN PALMITOYLOLEOYL PHOSPHATIDYLCHOLINE: PHOSPHATIDYLSERINE (90:10) LARGE UNILAMELLAR VESICLES, AS ASSAYED BY RESONANCE ENERGY TRANSFER BETWEEN FLUORESCENT PROBES

Example 5

A further experiment was carried out identical to that set forth in Example 3, with first maltose and then sucrose as the preserving agent. Results are set forth in Tables 4 and 5. As may be concluded from those tables, both maltose and sucrose provide good retention of encapsulated material following lyophilization.

Table 4

Method of Preparing LUV's	g Trehalose /g Lipid	Trehalose External	Trehalose Internal	%Retention
RD	0.05	-	+	3
RD	0.15	+	+	41
RD	0.25	+	+	88
RD	0.49	+	+	95
RD	0.64	+	+	100

Table 5

Method of Preparing LUV's	g Trehalose /g Lipid	Trehalose External	Trehalose Internal	%Retention
RD	0.07	-	+	20
RD	0.35	+	+	57
RD	0.49	+	+	89
RD	0.83	+	+	86
RD	1.15	+	+	91

Claims

1. A method for preserving liposomes, comprising:

providing initial liposomes, said liposomes having an initial quantity of water-soluble material encapsulated therein, said material including a first preserving agent;

contacting said initial liposomes with a second preserving agent in an aqueous solution; and,

lyophilizing said initial liposomes in the presence of said second preserving agent to form lyophilizates.

2. The method as in claim 1, further comprising recovering resultant liposomes from said lyophilizates by admixing said lyophilizates with an aqueous solution, wherein the resultant liposomes encapsulate at least about 25 wt.% of said initial quantity of encapsulated material.

3. The method as in claim 2, wherein said first preserving agent is trehalose, and said resultant liposomes encapsulate at least about 60% of said initial quantity of encapsulated material.

4. The method as in claim 1 or 2, wherein the weight ratio of said first and second preserving agents, combined, to lipid, ranges from about 0.1:1 to about 3.0:1.

5. The method as in claim 4, wherein said weight ratio is approximately 1.0:1.0.

6. The method as in claim 1 or 2, wherein said first preserving agent comprises at least two monosaccharide units.

7. The method as in claim 6, wherein said first preserving agent is selected from the group consisting of trehalose, maltose and sucrose.

8. The method as in claim 1 or 2, wherein said second preserving agent comprises at least two monosaccharide units.

9. The method as in claim 8, wherein said second preserving agent is selected from the group consisting of trehalose, maltose and sucrose.

10. A lyophilized composition useful in storing encapsulated material, prepared by the process comprising:

providing initial liposomes, said liposomes having an initial quantity of material encapsulated therein, said material including a quantity of trehalose;

contacting said initial liposomes with a quantity of trehalose in aqueous solution;

lyophilizing said initial liposomes in the presence of trehalose to form lyophilizates.

11. The composition of claim 10, wherein said encapsulated material includes a water-soluble therapeutic agent or biologically active compound.

12. The composition of claim 11, wherein said encapsulated material includes a macromolecule.

13. The composition of claim 12, wherein said encapsulated material includes a sympathomimetic drug, an antispasmodic, a vasodilator, an antineoplastic drug, RNA, DNA, an enzyme, or an immunoglobulin.

AMENDED CLAIMS

[received by the International Bureau on 30 May 1986 (30.05.86); original claims 1, 3, 4, 6-9 amended; other claims unchanged; new claims 14-18 added (4 pages)]

1. A method for preserving liposomes, comprising:

providing initial liposomes having lipid membranes, said liposomes having an initial quantity of water-soluble material encapsulated therein, said material including a first preserving agent comprising at least two monosaccharide units;

contacting said initial liposomes with a second preserving agent comprising at least two monosaccharide units in an aqueous solution; and,

lyophilizing said initial liposomes in the presence of said second preserving agent to form lyophilizates.

2. The method as in claim 1, further comprising recovering resultant liposomes from said lyophilizates by admixing said lyophilizates with an aqueous solution, wherein the resultant liposomes encapsulate at least about 25 wt.% of said initial quantity of encapsulated material.

3. The method as in claim 2, wherein said first preserving agent includes trehalose, and said resultant liposomes encapsulate at least about 60% of said initial quantity of encapsulated material.

4. The method as in claim 1 or 2, wherein the weight ratio of said first and second preserving agents, combined, to lipid of the lipid membrane, ranges from about 0.1:1 to about 3.0:1.

5. The method as in claim 4, wherein said weight ratio is approximately 1.0:1.0.

6. The method as in claim 1 or 2, wherein said first preserving agent comprises two monosaccharide units joined by a glycosidic linkage.

7. The method as in claim 1 or 2, wherein said first preserving agent is selected from the group consisting of trehalose, maltose and sucrose.

8. The method as in claim 1 or 2, wherein said second preserving agent comprises two monosaccharide units joined by a glycosidic linkage.

9. The method as in claim 1 or 2, wherein said second preserving agent is selected from the group consisting of trehalose, maltose and sucrose.

10. A lyophilized composition useful in storing encapsulated material, prepared by the process comprising:

providing initial liposomes, said liposomes having an initial quantity of material encapsulated therein, said material including a quantity of trehalose;

contacting said initial liposomes with a quantity of trehalose in aqueous solution;

lyophilizing said initial liposomes in the presence of trehalose to form lyophilizates.

11. The composition of claim 10, wherein said encapsulated material includes a water-soluble therapeutic agent or biologically active compound.

12. The composition of claim 11, wherein said encapsulated material includes a macromolecule.

13. The composition of claim 12, wherein said encapsulated material includes a sympathomimetic drug, an antispasmodic, a vasodilator, an antineoplastic drug, RNA, DNA, an enzyme, or an immunoglobulin.

14. The method as in claim 2 wherein said first preserving agent and said second preserving agent are in an effective amount to preserve the lipid membranes of the initial liposomes during formation of liphilosates and recovery of resultant liposomes therefrom.

15. The method as in claim 14 wherein both said first preserving agent and said second preserving agent include trehalose.

16. A preserved composition comprising:

liphilosates having a lipid component, a disaccharide component, and an initial quantity of an encapsulated component, the disaccharide component including trehalose and being in a weight ratio with respect to the lipid component from about 0.1:1 to about 3.0:1, the lipid component defining lipid membranes with an inner side and an outer side, the disaccharide component being present in the liphilosates at both the inner side and the outer side of the lipid membranes, and at least most of the initial quantity of encapsulated material being present

in the liphilosates at the inner side of the lipid membranes.

17. A composition as in claim 16 wherein the trehalose of the disaccharide component is present in the liphilosates at both the inner side and the outer side of the lipid membranes and is effective to retain most of the initial quantity of encapsulated material at the inner side of the lipid membranes during reconstitution of the liphilosates.

18. The composition as in claim 16 or 17 wherein the encapsulated material includes a water-soluble therapeutic agent, a biologically active compound, or a diagnostic agent, and the liphilosates are recoverable as liposomes encapsulating at least most of the initial quantity of encapsulated component by admixing said liphilosates with an aqueous solution.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/00016

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S. 424/38,85,94,DIG 7; 264/4.6 4.3; 435/177;/514/2

IPC[4] A01N 25/00,A61K 39/00,A01N 63/02,A61J 5/00,C12N 11/02,A61K 37/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	424/38, 85, 94, DIG. 7; 264/4.3, 4.6; 436/829; 435/177; 514/2; 935/54

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Chemical Abstracts 1967--> present
Biological Abstracts 1967--> present

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US., A, 4,411,894, Published 25 October 1983, Shrank et al., See column 2, lines 22-29	1,2,4-9
Y,P	US., A, 4,515,736, Published 07 May 1985, Deamer, See col. 4, lines 25-44	10-13
Y	N, Archives of Biochemistry and Biophysics, Vol. 220, No. 2, issued 01 February 1983, J. H. Crowe et al., "Preservation of Structural and Functional Activity in Lyophilized Sarcoplasmic Reticulum", pages 477-484.	3,9-13
Y	N, Biochimica et Biophysica Acta, Vol. 769, issued 11 January 1984, L. M. Crowe et al., "Effects of Carbohydrates on Membrane Stability at Low Water Activities", pages 141-150	3,9-13

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [3]	Date of Mailing of this International Search Report [3]
21 March 1986	01 APR 1986
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Shawn P. Foley

Form PCT/ISA/210 (second sheet) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	N, Chemical Abstracts, Vol. 97, No. 2, issued 1982, July 12 (Columbus, Ohio, U.S.A.), Jpn. Kokai Tokkyo Koho JP 82 46, 921, 17 March 1982, See page 370, column 1, the abstract no. 11851g.	1-13
Y	N, Chemical Abstracts, Vol. 99, No. 20, issued 1983, November 12 (Columbus, Ohio, U.S.A.), Tsyganenko, A.Ya. et al., "Preparation and low-temperature storage of rifampicin-containing liposomes" , See page 340, column 1, the abstract no. 163956z, Antibiotiki (Moscow) 1983, 28(8), 577-81 (Russ).	1-13
Y	N, Chemical Abstracts, Vol. 101, No. 24, issued 1984, December 12 (Columnbus, Ohio, U.S.A.), Crommelin, D.J.A. et al., "Stability of liposomes on storage: freeze-dried, frozen or as an aqueous dispersion", See page 307, column 2, the abstract no. 216308h, Pharm. Res. 1984, (4), 159-63(Eng).	1-13
P,X	N, Archives of Biochemistry and Biophysics, Vol. 242, No. 1, issued October 1985, L.M. Crowe et al., "Presevation of Freeze-Dried Liposomes by Trehalose", pages 240-247.	1-13.

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	N, Chemical Abstracts, Vol. 97, No. 2, issued 1982, July 12 (Columbus, Ohio, U.S.A.), Jpn. Kokai Tokkyo Koho JP 82 46, 921, 17 March 1982, See page 370, column 1, the abstract no. 11851g.	1-13

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ....., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.